INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print) 2222-5234 (Online)
http://www.innspub.net
Vol. 3, No. 7, p. 148-155, 2013

RESEARCH PAPER OPEN ACCESS

Biotransformation of ethyl *p*-methoxycinnamate from *Kaempferia galanga* L. using *Aspergillus niger*

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Key words: Biotransformation, *Kaempferia galanga* L., Ethyl *p*-methoxycinnamate, *Aspergillus niger*, NMR spectroscopy.

doi: http://dx.doi.org/10.12692/ijb/3.7.148-155 Article published on July 22, 2013

Abstract

Studies were carried out on the extraction of the rhizome of *Kaempferia galanga* L. using steam distillation and supercritical fluid extraction (SFE). After purification, the major compound of the *K. galanga* L. extract, ethyl *p*-methoxycinnamate (EPMC) was identified by NMR and MS spectrometry. Biotransformation of EPMC using Aspergillus niger afforded ethyl *p*-hydroxycinnamate in 24% yield.

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Introduction

Malaysian Zingiberaceae plants have been studied extensively. These include Zingiber, Alpinia and Kaempferia species (Omar *et al.*, 2013, De Pooter *et al.*, 1985, Samsudin *et al.*, 1989). *Kaempferia galanga* L. is one of the plants that mainly distributed in tropics and subtropics of Asia. Besides Malaysia, it can be found in Southern China, Indochina and India (Kanjanapothi *et al*, 2004). The rhizome of *K. galanga* L. plays an important role in native medicine as it possesses interesting biological activities such as expectorant, diuretic and carminative (Achuthan and Padikkala, 1997), antihypertensive (Othman *et al.*, 2002), larvicidal (Choochote, 1999), anticancer (Vimala *et al.*, 1999) and antiproliferative activity (Liu *et al.*, 2010).

Biotransformation involves the conversion of a compound by using enzymes either *in vivo* or *in vitro*. Microorganisms are one of the most efficient biocatalytic agents because of their abilities to metabolize a wide range of substrates. Biotransformation can be defined as chemical conversion of natural product substances by living organisms or enzyme preparation. In this case, microorganisms act as biocatalysts which possess several advantages over isolated enzymes such as cost effectiveness, ease of handling, simple operation, environmental friendly and several reactions in one pot (Choudhary *et al.*, 2004). Many fungi including species of *Aspergillus, Bacillus, Chaetosphaeria, Corynebacterium, Cunninghamella, Mucor, Penicillium,* and *Rhizopus* have been reported to have the ability to transform natural products. Srivastava *et al. (2008)* indicate that *A. flavus* can transform artemisinin to deoxyartemisinin that shows antibacterial activity against *Staphylococcus aureus, S. epidermidis* and *S. mutans* at a minimum inhibitory concentration (MIC) of 1 mg/mL compared to artemisinin whose MIC was >2 mg/mL. The study by Schmeda-Hirschmann *et al.* (2007) found that microbial transformation of diterpene, imbricatolic acid by *A. niger, R. nigricans* and *C. echinulata* afforded 1α-hydroxyimbricatolic acid and an epoxy derivative. The acid exhibited a moderate toxicity towards AGS cells and fibroblasts, with IC_{50} values of 307 and 631 μM, respectively. In another study done by Wang *et al.* (2009), biotransformation of the sesquiterpenoid (–)-maaliol by the fungus *M. plumbeus* afforded two new metabolites, (+)-7, 8-didehydro-9β-hydroxymaalioxide and (-)-7, 8-didehydro-1β-hydroxymaalioxide. So far the transformation of ethyl p- methoxycinnamate was done using microwave irradiation and its derivatives showed cycotoxic activity on WiDr cell. Ekowati *et al.* (2010) indicated that modification of carbonyl group of ethyl-p-methoxycinnamate afforded (E)-3-(4-methoxyphenyl)-N (phenylcarbomothioyl) acrylamide, (E)-3-(4-methoxyphenyl)-N-(4 methoxyphenylcarbomothioyl) acrylamide and (E)-3-(4-methoxyphenyl)-N-(4-methylphenylcarbomothioyl) acrylamide. However, none of these compounds showed cytotoxic effect on WiDr cell.

Therefore, this present study was initiated to transform the ethyl *p*-methoxycinnamate from *K. galanga* L by *A. niger* into other metabolites. It is believed to be useful in the discovery of new bioactive compound from natural sources and becomes forefront for the finding of new medicine which will benefit human health.

Materials and methods

Materials

The rhizomes of *Kaempferia galanga* L. were collected at different locations throughout Malaysia. The samples were then transported to the laboratory and stored at -20 °C prior to analysis.

Extraction of K. galanga and purification of ethyl p-methoxycinnamate

The fresh rhizomes of *K. galanga* L. were washed to remove the soil and cut into smaller pieces. Then, the rhizomes were extracted using steam distillation and supercritical fluid extraction (SFE) according to the previously reported method (Omar *et al.*, 2013). The extract and essential oil were collected and stored at -4°C for future analyses. For purification of ethyl-*p*-methoxycinnamate, the essential oils were mixed with

boiling water and then recrystalized at cold temperature (-4°C). After crystallization, the mixture was filtered through Whatman filter paper No 1 and then, the residue/crystal was kept in the desiccator for 24 hours and placed in reagent vial for further analysis.

Fungus culture preparation and biotransformation procedure.

Aspergillus niger fungal culture preparation

Pure fungal cultures of *A. niger* was obtained from (a) Microbiology Laboratory Kulliyyah of Science, International Islamic University Malaysia. *A. niger* pure fungus was streaked on Sabouraud Dextrose Agar slant (SDA) at 30 °C for a week and stored at 4 °C. After the cultivation, the well grown mycelia on the agar slants were inoculated into conical flask (250 ml) containing 100 ml of sterilized medium broth containing glucose, glycerol, peptone, yeast extract, KH_2PO_4, NaCl and distilled H_2O. The flask was incubated using an incubator shaker for two days (48 hr) at 120 rpm and 30°C. Then, mycelia suspensions were transferred into 17 flasks containing sterile broth medium (100 ml of each) and incubated for 24 hour using same condition (Choudhary *et al.*, 2004).

Biotransformation of the bioactive compounds

Ethyl-*p*-methoxycinammate (480 mg) was dissolved in dimethyl sulfoxide (DMSO) (24 ml) and distributed among 48 flasks containing 24 hour stage culture (10 mg/0.5 ml in each flask) and continuously shaken for 24 h using a rotary shaker (120 rpm) at 30 °C. At the same time, a control flask having substrate without fungus and a control flask containing fungus without substrate were analysed in order to check the substrate ability and to determine the endogenous metabolite, respectively.

Separation and purification of biotransformed product

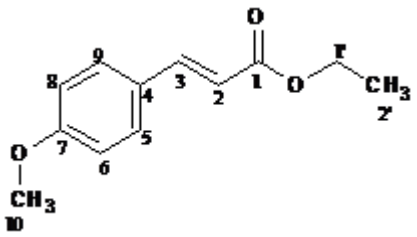
After incubation, the culture media and mycelium were separated using cotton in a funnel. Then, mycelium was washed with ethyl acetate (1.5 liter) while the culture media were extracted 3 times by ethyl acetate (2 liter). The combined organic extract was dried with anhydrous sodium sulphate (Na_2SO_4) and evaporated using a rotary evaporator. For the control group, the similar procedures were employed. All the residue from both experimental and control group were analysed by TLC to confirm the presence of biotransformed product. The biotransformed products were isolated by column chromatography using silica gel column (200-300 mesh) with hexane: ethyl acetate as solvent

Structural elucidation of the biotransformed product

Structures of the biotransformed product were elucidated on the basis of their GC-MS, ^{1}H NMR, and ^{13}C NMR analysis. Silica gel column chromatography was performed using silica gel G60, 70-230 mesh (Merck Ltd). NMR and MS were carried out at the Department of Chemistry, Universiti Kebangsaan Malaysia. Low resolution mass spectrometry (m/z) was carried out on a VG Biotech Quattro II triple quadrupole instrument. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker 400 MHz FT-NMR spectrometer; samples were dissolved in $CDCl_3$ or d_6-DMSO and chemical shifts (δ) are reported in ppm downfield of tetramethylsilane. Coupling constants (*J*) are quoted in Hz.

Results and discussion

Figures 1 and 2 showed the GC chromatogram of ethyl *p*-methoxycinnamate (1) and biotransformed product (2) respectively. As shown in Figure 1, there is only one chromatographic peak and identified as ethyl *p*-methoxycinnamate based on its mass spectrum in Figure 3 which included molecular ion, peak at 206.3422 allowing the possibility of molecular formula to be $C_{12}H_{14}O_3$. Other peaks were also observed at m/z: 178, 161 (100% base peak), 133, 118, 90, 77, 64 and 63. The structure of ethyl *p*-methoxycinnamate was determined by GC-MS and NMR spectroscopy and confirmed by comparing the NMR and GC-MS data with those from the literature. This structure is similar to the research done by Othman (1998)



Compound 1

Table 1. ^{1}H- NMR, and ^{13}C-NMR data of ethyl p methoxycinnamate (600 MHz, CD_2Cl_2).

Position	^{13}C-NMR	^{13}C-NMR (Rozana, 1998)	^{1}H- NMR	^{1}H- NMR (Rozana, 1998)
1	167.55, C	167.18	-	-
2	116.28, CH	115.68	6.3 (1H, d, J = 15.6 Hz)	6.31 (1H, d, J = 15.9 Hz)
3	144.45, CH	144.12	7.6 (1H, d, J = 16.2 Hz)	7.63 (1H, d, J = 16.1 Hz)
4	127.65, C	127.13	-	-
5, 9	130.19, CH	129.57	7.5 (2H, d, $J_5 = J_9$ = 9.0 Hz)	7.46 (2H, d, $J_5 = J_9$ = 9.0 Hz)
6, 8	114.77, CH	114.22	6.9 (2H, d, $J_6 = J_8$ = 8.4 Hz)	6.89 (2H, d, $J_6 = J_8$ = 8.8 Hz)
7	161.28, C	161.25	-	-
10	55.89, OCH_3	55.22	3.8 (3H, s)	3.81
1’	60.77, CH_2	60.18	4.2 (2H, q)	4.25
2’	14.60, CH_3	14.25	1.2 (3H, t)	1.32

Table 2. ^{1}H- NMR and ^{13}C-NMR, data of compound **2** (600 MHz, CD_2Cl_2).

Position	^{13}C-NMR	^{13}C-NMR (Barbosa-Filho *et al.*, 2004)	^{1}H- NMR	^{1}H- NMR (Barbosa-Filho *et al.*, 2004)
1	167.42, C	167.14	-	-
2	115.55, CH	115.09	6.3 (1H, d, J = 15.6 Hz)	6.53 (1H, d, J=15.9 Hz)
3	144.18, CH	144.87	7.6 (1H, d, J = 16.2 Hz)	7.89 (1H, d, J=15.9 Hz)
4	127.07, C	125.94	-	-
5, 9	129.94, CH	130.43	7.5 (2H, d, $J_5 = J_9$ = 9.0 Hz)	7.55 (2H, d, J=8.6 Hz)
6, 8	115.80, CH	116.66	6.9 (2H, d, $J_6 = J_8$ = 8.4 Hz)	7.11 (2H, d, J=8.6 Hz)
7	157.98, C	161.26	-	-
1’	60.77, CH_2	60.01	4.2 (2H, q)	4.20 (2H, q, J=7.1 Hz)
2’	14.60, CH_3	14.29	1.2 (3H, t)	1.15 (3H, t, J=7.1 Hz)

From the ^{1}H- NMR result in table 1, triplet corresponding to three protons at 1.2 ppm belonged to methyl adjacent to an ethyl. While a quartet at 4.2 ppm assigned to the two protons on the ethyl group which adjacent to methyl and bonded to the oxygen atom. Thus, highly deshielding position of the proton could be because of the neighbouring to oxygen atom. Electronegative atoms are said to deshield the proton and cause it to have a higher chemical shift, moving it to the downfield. Meanwhile, another strong peak at 3.8 ppm corresponding to three protons due to the methoxy directly attached to the aromatic ring. The

dublet resonating at 6.3 ppm (1H, d, J = 15.6 Hz) which belonged to proton attached to C-2 position while proton attached to C-3 position futher downfield at 7.6 ppm (1H, d, J = 16.2 Hz) .The strong deshielding effect at this proton due to the fact that it was at a β position to an ester. A doublet at 6.9 ppm (2H, d, $J_6 = J_8$ = 8.4 Hz) corresponding to two proton which bonded to C-6 and C-8 while doublet at 7.5 ppm (2H, d, $J_5 = J_9$ = 9.0 Hz) was assigned to two proton which attached to C-5 and C-9. Both of doublet attached to the aromatic ring showed high deshielding position because it have large magnetic field compared to proton elsewhere in the molecule. Aromatic ring protons will therefore resonate at higher frequency and exhibit a downfield shift.

Fig. 1. GC chromatogram of Ethyl *p*-methoxycinnamate from *Kaempferia Galanga* L.

The ^{13}C-NMR result of this compound showed that the first signal at 14.60 ppm indicated methyl carbon. Meanwhile, ethyl carbon which adjacent to methyl and oxygen atom gave peak at downfield position due to deshielding effect cause by neighbouring oxygen atom which more electronegativity. The signal at 55.89 ppm corresponded to the methoxy carbon which directly attached the aromatic ring. The strong peak at 167.55 ppm corresponded to the quaternary C-1 that bonded to the carbonyl group. Hence it has high deshielding position due to the oxygen atom adjacent to this carbon. Carbon signal at 161.95 ppm assigned to C-7 which are also quaternary carbon. Based on COSY result in the table 1, the peak at 114.77 ppm and 130.19 ppm were adjacent to each other and assigned to C-6,8 and C-5,9 respectively. Lastly, the signal at 127.65 corresponded to the C-4.These finding confirmed the starting material is ethyl *p*-methoxycinnmate.

Fig. 2. GC chromatogram of biotranformed product by *Aspergillus Niger* bacterium.

After incubation for 24 h, the biotransformation product was isolated and identified based on GC-MS and NMR spectroscopy. Microbial transformation of **1** by *A. niger* afforded a main compound, identified as ethyl *p*-hydroxycinnamate (**2**). The molecular formula of **2** proved to be $C_{11}H_{12}O_3$ from GC-MS, ^{1}H-NMR and ^{13}C-NMR data which included a molecular ion, peak at m/z: 192.3220. Other peaks were also observed at

m/z: 177, 164,147 (100% base peak), 120, 91, 77, 65 and 63.

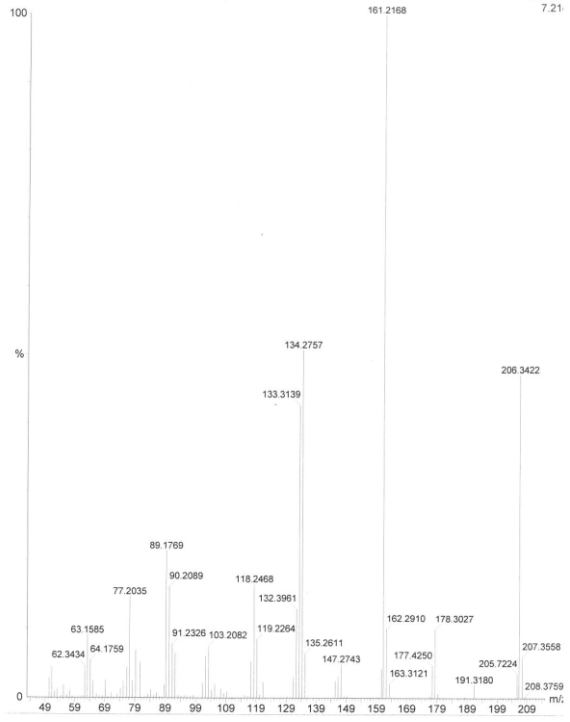


Fig. 3. Mass Spectrum of ethyl *p*-methoxycinnamate.

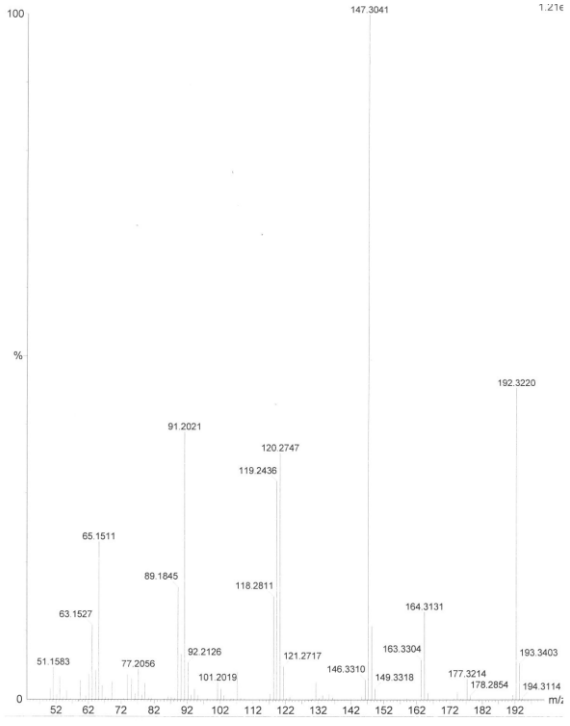
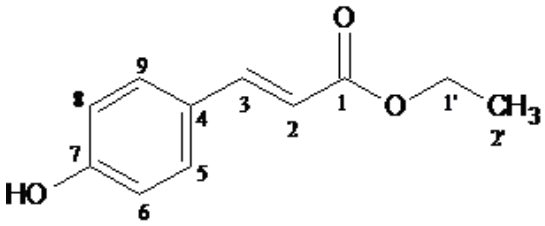


Fig. 4. Mass Spectrum of biotranformed product by *Aspergillus niger* bacterium.



Compound **2**

The 1H-NMR spectrum of compound **2,** was closely related to **1**, differing mainly by the presence of OH group, instead of a methoxy group which bonded to the aromatic ring. However, the signal of proton in OH was absence in the spectrum. It was due to exchangeable protons with the solvent used. OH signals move downfield in H-bonding solvents. Rapid exchange of this hydrogen with heavy water caused the low field signal to disappear in spectrum.

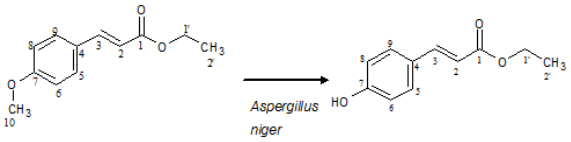


Fig. 5. Biotransformation of ethyl *p*-methoxycinnamate into ethyl *p*-hydroxycinnamate by *Aspergillus niger*.

In the 13C-NMR spectrum of **2** also was close to **1**, differing mainly by the loss of one carbon in compound **2**. The strong peak observed in C-7 which gave signal at 157.98 ppm in the aromatic ring which directly bonded to OH group. From the COSY result of compound **2**, the signal at 144.18 ppm corresponds to the C-3 which adjacent to the C-2 that gave signal peak at 115.55 ppm. While the signal peaks at 115.80 ppm and 129.94 ppm were adjacent to each other and assigned to C-6, 8 and C-5, 9 respectively. Lastly, the signal at 127.07 ppm corresponded to the C-4. In figure 5, it confirmed that hydroxyl group was attached to the C-7 position of **2** instead of methoxy group in **1**. The NMR spectral data of compound **2** are summarized in the Table 2. These finding presented a clear correlation to support the proposed structure and identified by comparing their spectra with literature. (Barbosa-Filho *et al.*, 2004). Ethyl *p*-hydroxycinnamate (**2**) has been reported as good antimicrobial agent against Gram positive and Gram

negative bacteria, alcohol-acid bacterium and fungi (Barbosa-Filho *et al.*, 2004). The present of hydroxyl group in structure may increase the antimicrobial activities of compound. (Gouiric *et al.*, 2004). Until now, ethyl *p*-hydroxycinnamate was reported only from extraction of natural products (Barbosa-Filho *et al.*, 2004; De Abreu *et al.*, 2010).

In conclusion, we develop a new method for preparation of ethyl *p*-hydroxycinnamate by transformation of ethyl *p*-methoxycinnamate using *A. niger*. The yield of biotransformation product of **2** was 24%.

Acknowledgement

The authors would like to express greatest appreciation and gratitude to the International Islamic University of Malaysia (IIUM) and The Ministry of Higher Education (MOHE) for financial support (RMGS). Also thank to all Kulliyyah of Science (KOS) and IIUM staff for technical supports.

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